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Characterisation of Cadmium Chloride Induced Molecular and Functional Alterations in Airway Epithelial Cells

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Key Words

Calu-3 • $CdCl_2$ • Lung toxicity • *In vitro* • TEER • MT1X • HSP70 • HMOX-1 • Antioxidants

Abstract

Epidemiological studies show that cadmium (Cd) exposure causes pulmonary damage, such as emphysema, pneumonitis, and lung cancer. However, the mechanisms leading to pulmonary toxicity are not yet fully elucidated. The aim of this study was to further investigate cadmium chloride ($CdCl_2$) induced toxicity using Calu-3 cells as an *in vitro* model of human bronchial epithelial cells. $CdCl_2$ induced effects following either apical or basolateral exposure were evaluated by Neutral Red Uptake (NRU), Trans-Epithelial Electrical Resistance (TEER), and alteration in Metallothionein 1X (MT1X), Heat shock protein 70 (HSP70), and Heme oxygenase 1 (HMOX-1) genes. $CdCl_2$ exposure resulted in a collapse of barrier function and the induction of MT1X, HMOX-1 and HSP70 genes, prior to alterations in cell viability. These effects were more pronounced when the exposure was from the basolateral side. Co-administration of N-Acetylcysteine (NAC) exerted a strong protective effect against $CdCl_2$ induced barrier damage and stress related genes, while other antioxidants only attenuated $CdCl_2$ induced HSP70 and HMOX-1 and showed no protective effect on the barrier collapse. These findings indicate that $CdCl_2$ exposure is likely to impair Calu-3 barrier function at non cytotoxic concentrations by a direct effect on adherens junction proteins. The protective effect of NAC against $CdCl_2$ induced MT1X, HSP70 and HMOX-1 genes, demonstrates an anti-oxidant effect of NAC in addition to Cd chelation.

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Introduction

Cd is a toxic heavy metal that naturally occurs in ores together with zinc, cooper and lead, and is widely used in industry for different purposes such as colour pigment, anticorrosive agent and stabilizer in PVC products and most commonly in re-chargeable nickel-cadmium batteries. Moreover, Cd is known to have a half-life in humans of more than 20 years. Human intoxication is mainly caused by cigarette smoke but also by other

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sources such as contaminated water, food and air. The main routes for exposure are the pulmonary and digestive system and the primary target organs are the liver, the kidney, the respiratory system, the reproductive system and the skeletal system depending on the dose and the time of exposure [1]. In addition, inhalation of Cd dusts or exposure to air contaminated with Cd can result in acute injury such as oedema, or in chronic injuries like emphysema, pulmonary fibrosis or adenocarcinomas [2-4].

The mechanisms of Cd toxicity are not yet fully understood, several publications reported on interference with essential metals, induction of oxidative stress, disruption of cadherins, inhibition of DNA repair and interference with apoptosis (See reviews: [5-8]). Also some of the molecular sensors of Cd intoxication are known such as metallothionein (MT), heme oxygenase-1 (HMOX-1), and heat shock protein 70 (HSP70). It is thought that the induction of these and other proteins play a role in cell survival [9-15].

The airway epithelium serves as a biological barrier that protects against the entry of exogenous substances. Therefore, the ability to form tight junction *in vitro* is important in the assessment of pulmonary toxicity. Calu-3, a very well characterized cell line derived from a human bronchial adenocarcinoma [16], is known to have retained a number of the main characteristics of the native airway epithelium such as tight junctions formation, presence of secretory granules, functional cystic fibrosis transmembrane conductance regulator (CFTR), and P-glycoprotein activity. Moreover, Calu-3 cells have been reported to be able to produce mucous when cultured at the air-interface [17-27].

The aim of this study was to further elucidate the mechanisms of $CdCl_2$ toxicity on the *in vitro* Calu-3 polarized barrier model and to assess the impact of apical and basolateral $CdCl_2$ exposure on transepithelial electrical resistance (TEER) and mRNA alterations of HMOX-1, MT1X and HSP70.

Materials and Methods

Materials

All compounds unless otherwise mentioned, were purchased from Sigma (Milan, Italy).

Experimental Design

Fig. 1 shows a schematic representation of the different experiments performed in Calu-3 cells. The experimental conditions are described in detail in the following paragraphs.

Calu-3 Cells in Culture

Maintenance - Calu-3 cells were purchased from the American Type Culture Collection (ATCC, USA). The cells were routinely maintained in Minimum Essential Medium Eagle (MEM) supplemented with 10% Fetal bovine serum (FBS, Lonza, Milan, Italy), 0.1 mM Non-essential amino acids, 1 mM Sodium Pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in 75 cm^2 (Corning, Pero, Italy) tissue culture flasks in a humidified 37°C, 5% CO_2 incubator and were subcultured when 90% confluence was reached. Prior to experiments, cells were seeded either in 96-well plates (Corning) or on transwells membrane filters (0.33 cm^2 polyester, 0.4 µm pore size, Corning). Cells were used between passages 20 and 45.

Calu-3 in 96 well plates - Calu-3 cells were seeded in 96 well plates at a cell density of $3x10^5$ cells/ml (100 µl/well) and cultured for 14 days.

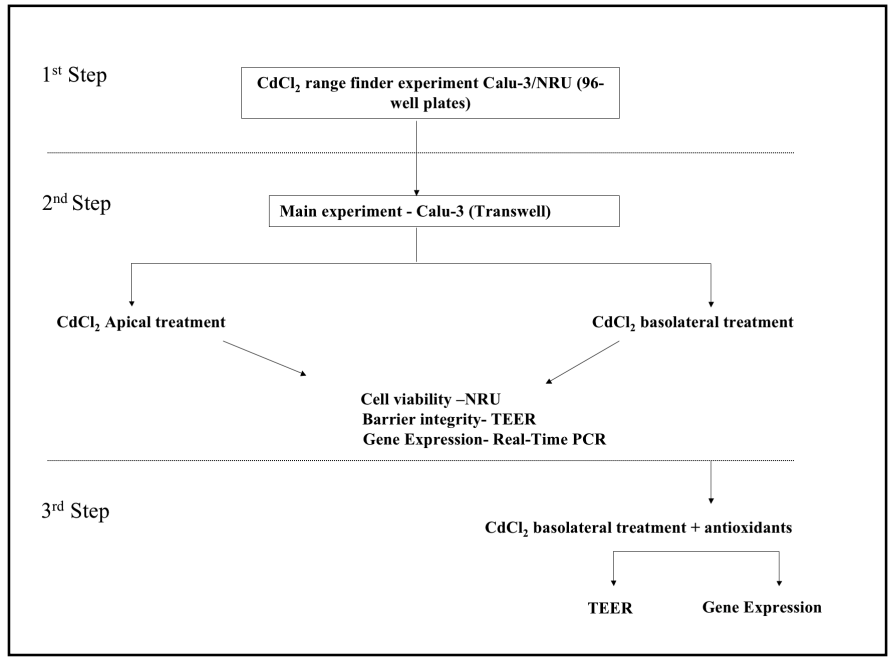
Calu-3 on transwells - Calu-3 were seeded on the filters at a cell density of $1x10^5$ cells/cm^2 in 250 µl of complete medium. One ml of medium was added to the bottom compartment. On the following day, Air Interfaced Culture (AIC) was established by removal of the medium from the apical compartment. The medium in the basolateral compartment was replaced every second day. The cells seeded on transwells were grown for 14 days in a 37°C, 5% CO_2 humidified incubator, in order to reach a tight and stable barrier with trans-epithelial electrical resistance (TEER) values higher than 150 Ω cm^2. Day 14 and TEER values >150 Ω cm^2 were selected as minimal requirements to consider the model suitable for the study.

Microscopy

For electron microscopy, Calu-3 cells were washed twice for 5 min in phosphate buffered saline solution (PBS) at 37°C, then fixed with 4% formaldehyde (freshly depolymerised from paraformaldehyde) and 1% glutaraldehyde, in PBS for 45 min at 37°C. Cells were then washed twice in PBS (5 min each) at room temperature, and postfixed in 1% OsO_4. Samples for Scanning Electron Microscopy (SEM) were dehydrated in graded series of methanol and submitted to critical point drying using CO_2. The dried specimens were sputter coated with gold-palladium and examined with a JEOL SEM (JSM-20). Samples for Transmission Electron Microscopy (TEM) were dehydrated in acetone and embedded in Durcupan ACM (Fluka, Switzerland) and examined with a Zeiss TEM (Zeiss EM-10).

For confocal microscopy, cells were washed in PBS and fixed in ice cold methanol for 20 min at -20°C. After fixation, cells were washed and incubated with 0.2% Triton X-100 for 10 min at room temperature, then washed and blocked for 30 min with 10% of goat serum. Cells were incubated with mouse anti-ZO-1 (5 µg/ml in 1% goat serum) or mouse anti- E- cadherin (2 µg/ml in 1% goat serum) for 2 h and washed 3 times. Goat anti-mouse IgG Alexa 546 (1:1000 in 1% goat serum) was applied for 2 h. Cells were washed, and then transwells filter membrane was cut out from the plastic support and mounted on a microscopic slide in anti-fade medium (Invitrogen, San Giuliano Milanese, Italy). Slides were stored at 4°C in a humid atmosphere until observation under confocal laser scanning microscope (BioRad Radiance MP2000, USA).

Fig. 1. Schematic representation of the experimental steps followed in the study.



Cadmium Chloride Exposure

Twenty-four hours before treatment, FBS in medium was reduced from 10 to 0.1%. A stock solution of $CdCl_2$ (10 mM) was prepared in distilled water and stored in aliquots at -20°C. On the day of experiment, working concentrations of $CdCl_2$ (1-300 µM) were freshly prepared in 250 µl PBS (apical exposure) or in 1 ml medium containing 0.1% FBS (basolateral exposure).

Antioxidants pre-treatment

To test the protective effect of antioxidants, 1 h prior to the treatment with $CdCl_2$, Calu-3 cells were incubated with Catalase (CAT, 1500 U/ml), Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP, 100 µM), α-Tocopherol (Vitamin E, 50 µM), or N-Acetyl-L-cysteine (NAC, 5 mM). All antioxidants were freshly diluted in 1 ml medium containing 0.1% FBS (basolateral treatment). $CdCl_2$ (10 µM) was directly added to the cells in the presence of the antioxidants.

Trans-Epithelial Electrical Resistance (TEER) Measurement

To assess the integrity of the barrier, TEER was measured by placing the transwells in an Endohm-6 chamber electrode (World Precision Instruments, Berlin, Germany) connected to an EVOM voltohmmeter (World Precision Instruments). Before measuring, 1.4 ml of pre-warmed medium was added to the chamber electrode. Pre-warmed PBS (250 µl) was added at the apical compartment and aspirated back immediately after the measurement. For each set of experiments, TEER was also measured in a cell-free filter (Blank) and the value obtained was subtracted from raw TEER data of the cell seeded filters. TEER expressed as Ω cm^2 was obtained by multiplying TEER values (corrected by the background resistance of the blank filter) by the surface area of the filter (0.33 cm^2).

Neutral Red Uptake Assay (NRU)

Cell viability was assessed both in 96 well plates and in transwells plates by NRU as previously described [28]. Briefly, after exposure to $CdCl_2$, cells were washed with 250 µl PBS and incubated for 3 h at 37°C, 5% CO_2, with 250 µl of medium containing 0.1% FBS and 25 µg/ml neutral red. Medium was removed and the cells were washed with 250 µl of PBS. Neutral red destaining solution (5 parts ethanol, 4.9 parts distilled water and 0.1 part glacial acetic acid) was added (200 µl per filter and 100 µl per well in 96 well plates) and the plates were placed on an orbital shaker for 45 min. Absorbance was recorded at 540 nm using a spectramax 250 plate reader (Molecular device, Sunnyvale CA, USA). For experiments performed on transwells, the volume in the apical compartment (200 µl) was transferred to a 96 well plate for the absorbance recording. The optical density (OD) values obtained were then used to generate a concentration-response curve from which the EC_{50} value was further calculated using the Hill function.

Gene Expression Analysis

Cells were lysed and total RNA was extracted according to the instructions of applied RNeasy Mini Kit (Qiagen, Milan, Italy). For reverse transcriptase reaction (final volume of 20 µl), 25 ng/µl RNA were incubated with 2.5 mM PCR Nucleotide Mix (Promega, Milano, Italy) and 12.5 ng/µl random primers (promega) for 5 min at 65°C. M-MLV buffer (Promega) was added to the samples together with 10 U/µl of M-MLV reverse transcriptase (Promega) and 2 U/µl of RNaseOUT (Invitrogen). The termocycling conditions of the reverse transcription reaction were the following: annealing at 25°C for 10 min, cDNA synthesis at 37°C for 1 h and enzymes inactivation at 70°C for 15 min. mRNA levels of different genes were determined by TaqMan real-time PCR using ABI PRISM 7000 sequence de-

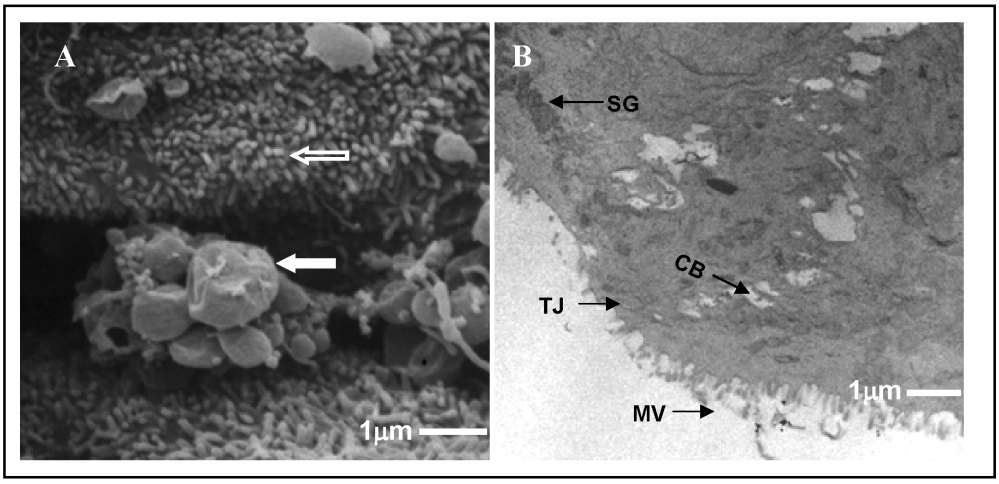


Fig. 2. Electron micrographs of Calu-3 cells cultured at the air interface. SEM image (A) shows apical microvilli (open arrow), and exuding mucous (filled arrow). TEM image (B) shows some secretory granules (SG), tight junctional complexes (TJ), microvilli (MV), and cell boundaries (CB).

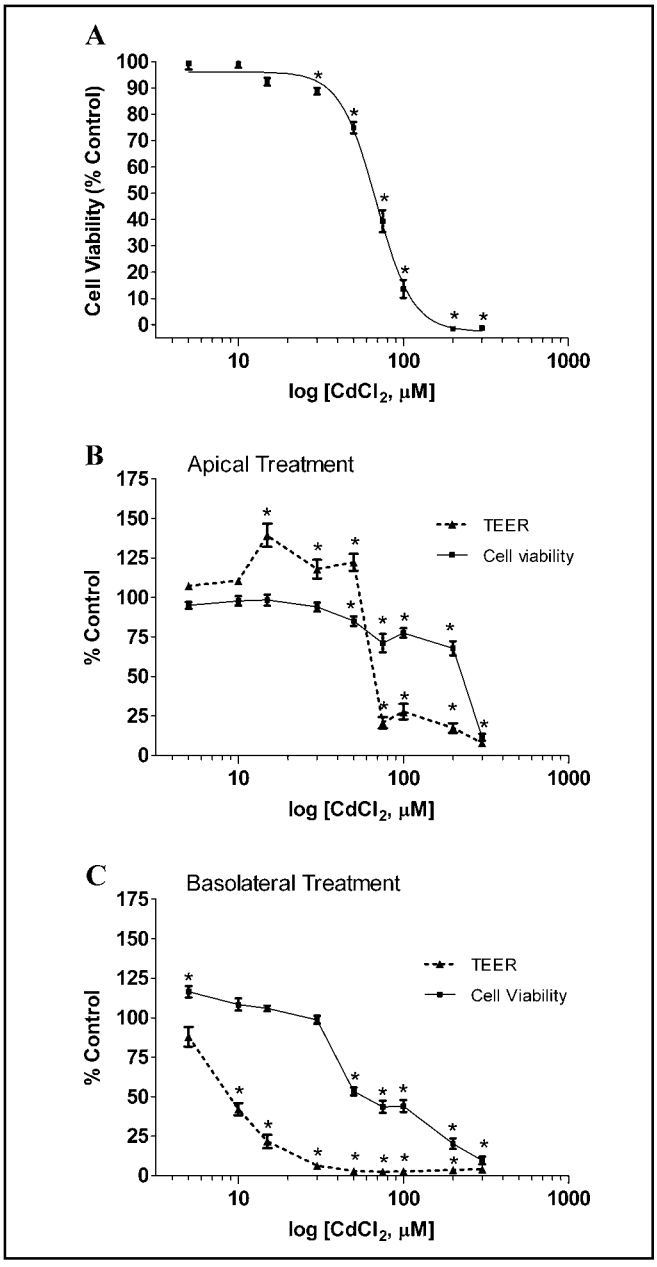


Fig. 3. $CdCl_2$ concentrations range finding and the concentration dependent effect of apical or basolateral exposure on TEER and NRU. A: Cells were grown in 96-well plates for 14 days and treated for 24 h with different concentrations of $CdCl_2$ (1-300 µM), cell viability was then assessed by NRU assay. B-C: Cells grown on transwells for 14 days were treated for 24 h with $CdCl_2$ applied either to the apical (B) or to the basolateral (C) compartment. Following exposure, TEER was measured and cell viability was assessed by performing NRU assay. The results were expressed as percentage (%) of control (untreated cells) ± SEM of 3 independent experiments (3 replicates each). *Significant difference with respect to untreated control ($P < 0.05$).

tection system according to the instructions of the manufacturer (Applera Italia, Monza, Italy). The primers used were (gene symbol, assay ID): β-actin (ACTB, Hs99999903_m1), Metallothionein 1X (MT1X, Hs00745167_sH), Heat shock 70KDA protein (HSP70, Hs00271229_s1), Heme oxygenase (decycling) 1 (HMOX-1, Hs00111251_m1). Data analysis was performed by ΔΔCt method with β-actin as the house-keeping gene.

Statistical Analysis

Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for data plotting, non-linear regression and statistical analysis. All results are expressed as mean ± S.E.M. Differences were considered significant when P-values from one-way ANOVA analysis were less than 0.05 ($P<0.05$).

Results

Morphology of Calu-3 Cells Cultured at the Air-Interface

For our studies, Calu-3 cells were seeded on the apical side of transwells filters and AIC was obtained by aspirating the medium from the apical side at day 1 after

seeding. Cellular morphology was assessed by electron microscopy after 14 days in culture. SEM and TEM revealed the presence of apical microvilli (Fig. 2A, 2B), with apical projections on the surface of some of the cells. TEM analysis did not confirm the presence of kinocilia. The presence of mucous secreted by Calu-3 cells was shown by SEM (Fig. 2A) and was confirmed also by Alcian blue staining for the acidic glycoproteins typically found in mucous (data not shown).

Tight junctions were observed by TEM (Fig. 2B) and the expression profile of the tight junction (TJ) protein ZO-1 and adherence junction (AJ) protein, E-cadherin showed a regular staining pattern along cell-cell interface (Fig. 5, control).

Effect of $CdCl_2$ on Cell Viability

Preliminary, range finding experiments were performed in 96 well plates. Calu-3 cells were treated with $CdCl_2$ (1-300 μM) for 24 h. As measured by the NRU assay a $CdCl_2$ concentration dependent decrease in cell viability was observed (Fig. 3A), and an EC_{50} value of 68.6 μM was calculated. The lowest $CdCl_2$ concentration exerting a significant cytotoxic effect was 30 μM with a decrease of 12% in NRU.

A similar set of experiments were conduced for filter grown Calu-3 cells and $CdCl_2$ was applied on either the apical or basolateral side. A concentration dependent decrease in cell viability was observed after both types of exposure (Fig. 3B, C). The calculated EC_{50} value for basolateral treatment was 50.3 μM, whereas EC_{50} value for apical treatment was between 200 and 300 μM.

Effect of $CdCl_2$ on the Integrity of the Barrier

Apical treatment with $CdCl_2$ caused an increase in TEER values with concentrations up to 50 μM. Concentrations higher than 75 μM $CdCl_2$ decreased TEER with an EC_{50} value of 69.8 μM (Fig. 3B). Basolateral exposure resulted in a decrease in TEER values beginning at 5 μM with an EC_{50} of 7.8 μM (Fig. 3C). Experiments were also conducted to investigate the kinetics of $CdCl_2$ induced alterations in TEER. Basolateral $CdCl_2$ exposure resulted in a more sensitive and quicker collapse in TEER than apical exposure (Fig. 4).

Effect of $CdCl_2$ on the Localization of ZO-1 and E-Cadherin

To determine whether the exposure of the cells to $CdCl_2$ affects the organization of the tight and adherens junction proteins, immunoflurorescence for ZO-1 and E-cadherin was performed. A 24 h apical exposure to 10 μM $CdCl_2$ did not affect ZO-1 or E-cadherin expression patterns (Fig. 5). However, 10 μM $CdCl_2$ applied basolateraly resulted in the loss of cell-cell border ZO-1 staining in most cells and caused an increased cytosolic expression of E-Cadherin (Fig. 5).

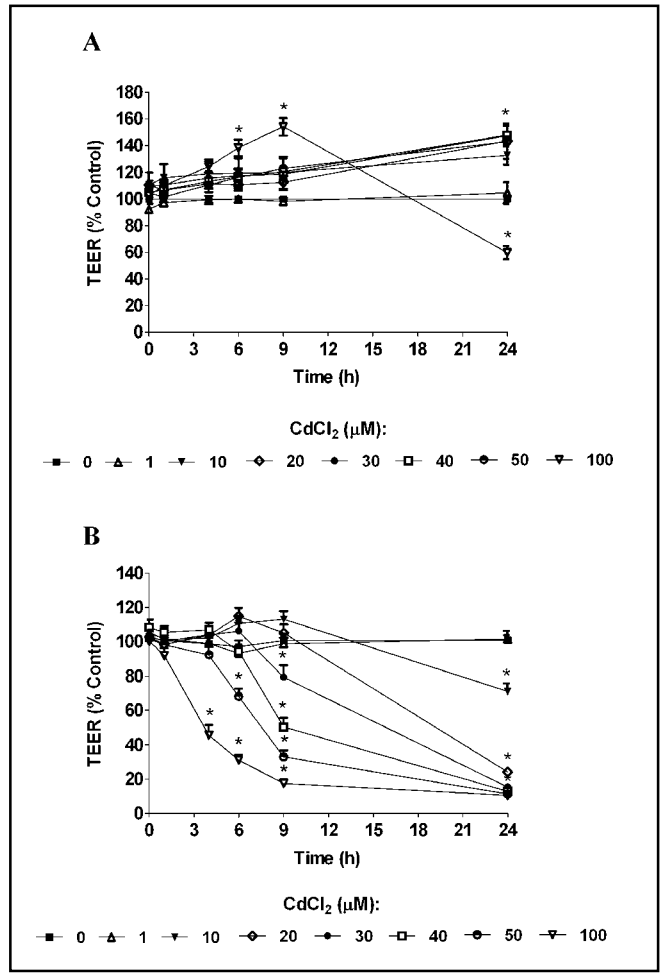


Fig. 4. Time dependent effect of $CdCl_2$ on TEER. Cells were treated with different concentrations of $CdCl_2$ (1-100 μM) applied either to the apical (A) or to the basolateral compartment (B). Following exposure, TEER was monitored during time at 1, 4, 6, 9 and 24 h. The results were expressed as percentage (%) of control (untreated cells) ± SEM of 2 independent experiments (3 replicates each). *Significant difference with respect to the untreated control ($P < 0.05$).

$CdCl_2$ Induced Gene Changes

MT1X and HMOX-1 mRNA levels were up-regulated in a concentration-dependent manner after both apical and basolateral treatment, however, basolateral

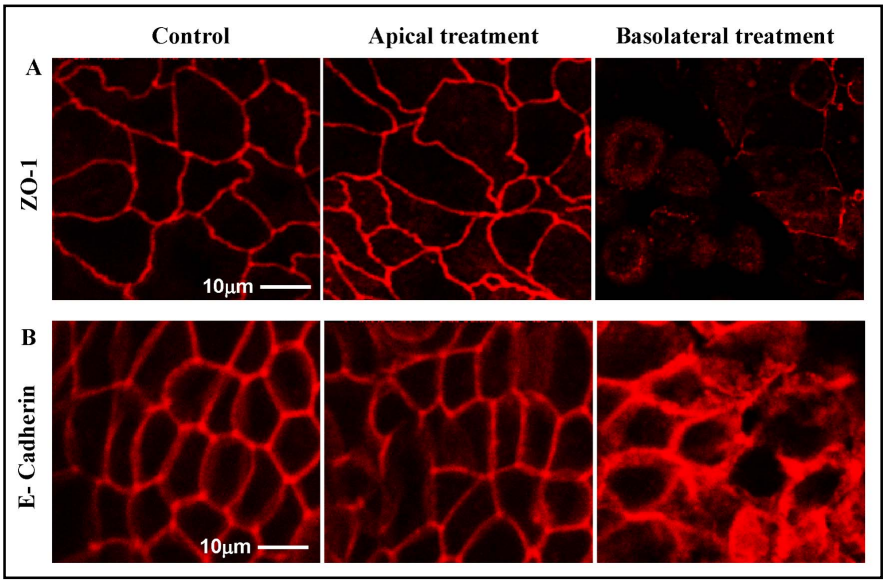


Fig. 5. Effect of $CdCl_2$ on the immunofluorescent pattern of ZO-1 and E-Cadherin. Calu-3 cells were treated for 24 h with $CdCl_2$ (10 µM) applied either to the apical or to the basolateral compartment. Following exposure, cells were fixed with ice cold methanol, blocked in goat serum and incubated with antibodies against ZO-1 (A) or E-cadherin (B). Cells were then incubated with secondary antibody, rinsed and mounted on a glass slide and viewed using a confocal microscope.

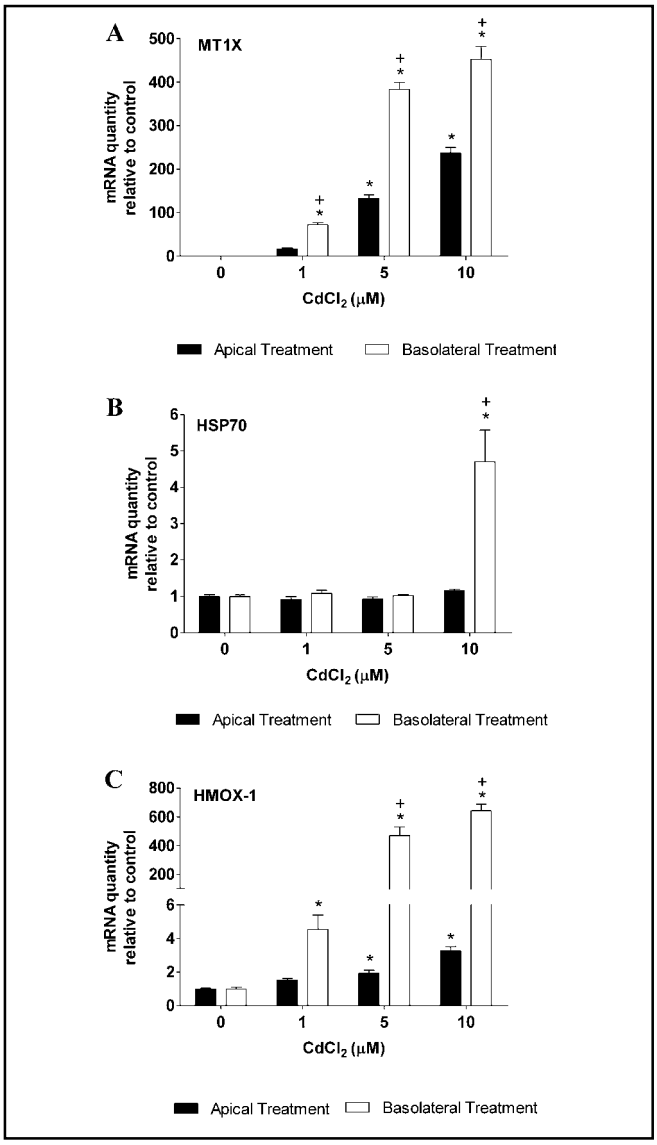


Fig. 6. Effect of $CdCl_2$ on MT1X, HSP70, and HMOX-1 mRNA levels. Non cytotoxic concentrations of $CdCl_2$ (1-10 µM) were applied for 24 h either to the apical or to the basolateral compartment. Total RNA was then isolated and transcript levels of MT1X (A), HSP70 (B), and HMOX-1 (C) were determined by real-time PCR and normalized to internal house keeping control gene levels (β-actin). The results were expressed relatively to control (untreated cells) ± SEM of 3 independent experiments (2 replicates each). *Significant difference from the untreated control, +Significant difference of basolateral versus apical treatment at the same concentration of $CdCl_2$ ($P < 0.05$).

exposure had a more pronounced effect (Fig. 6A, C). Maximal induction of MT1X mRNA levels was obtained at 10 µM $CdCl_2$ with 237 ± 13 -fold over control upon apical exposure and 452 ± 30 -fold over control upon basolateral exposure, $P<0.001$ (Fig. 6A). For HMOX-1, maximal mRNA levels were achieved at 10 µM $CdCl_2$ with 3.3 ± 0.2 -fold over control upon apical exposure and 641 ± 47 -fold over control upon basolateral exposure, $P<0.001$ (Fig. 6C). Up-regulation of

HSP70 mRNA was observed upon basolateral exposure with 10 µM $CdCl_2$ (4.7 ± 0.9 -fold over control, $P<0.001$) (Fig. 6B).

Effect of Antioxidants

Cd is thought to induce oxidative stress through indirect elevation of reactive oxygen species levels and inhibition of antioxidant enzyme activity. Therefore, the effect of antioxidant exposure on $CdCl_2$ induced barrier collapse was investigated. The cells were incubated with

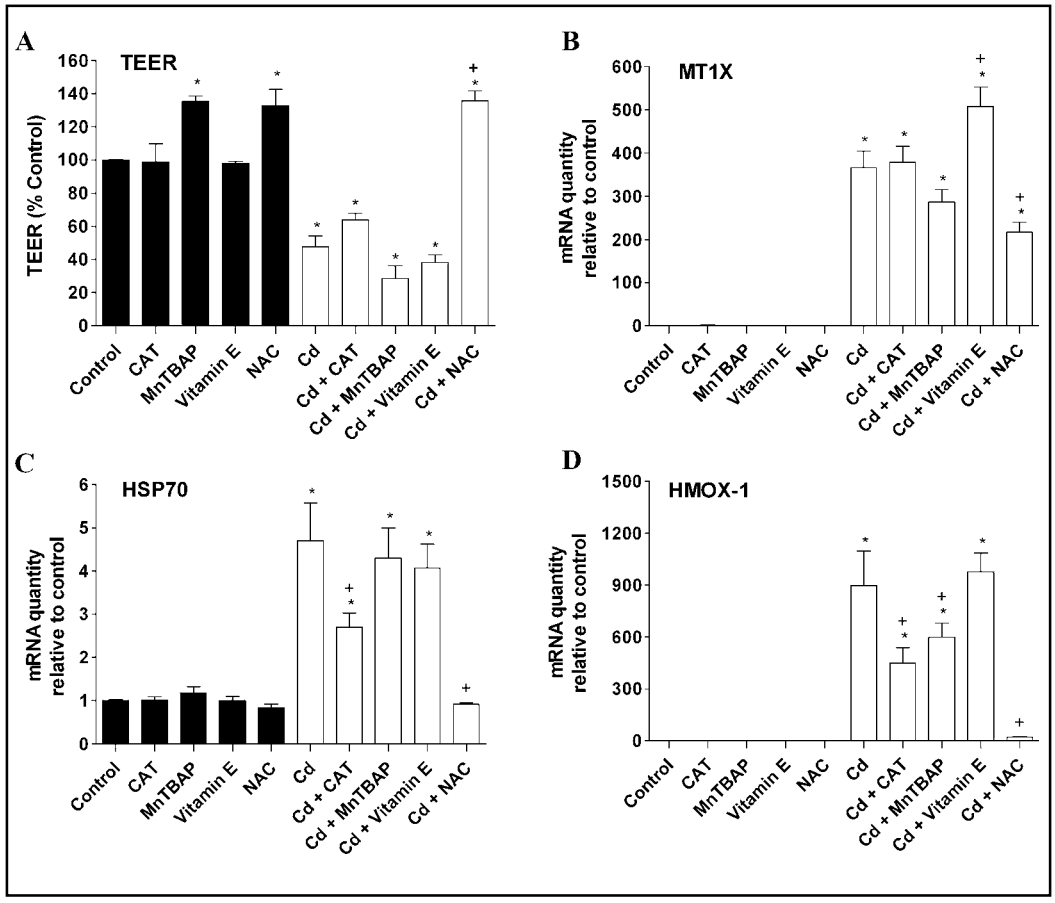


Fig. 7. Protective effect of antioxidants on $CdCl_2$ induced damage to the barrier and up-regulation of stress related genes. Cells were pre-treated for 1 h with different antioxidants before addition of $CdCl_2$ (10 μM) to the basolateral compartment, after 24 h exposure TEER was measured (A), total RNA was isolated and transcript levels of MT1X (B), HSP70 (C), and HMOX-1 (D) were determined by real-time PCR and normalized to internal house keeping control gene levels (β-actin). The results were expressed as percentage (%) of untreated control ± SEM of 3 independent experiments (2 replicates each). *Significant difference from the untreated control, +Significant difference from treatment with $CdCl_2$ only ($P < 0.05$).

different antioxidants for 1 h prior to basolateral addition of 10 μM $CdCl_2$. Antioxidants alone did not cause a decrease in TEER (Fig. 7A). Pre-treatment with CAT, MnTBAP and Vitamin E, had no effect on $CdCl_2$ induced TEER decrease. In contrast, pre-treatment with 5 mM NAC completely protected the cells from $CdCl_2$ induced damage to the barrier (Fig. 7A).

In addition, the capability of different antioxidants to inhibit the over-expression of MT1X, HSP70 and HMOX-1 caused by basolateral treatment with $CdCl_2$ was investigated. Only NAC attenuated $CdCl_2$ induced MT1X expression (reduced by 37% ± 17, $P<0.001$). Vitamin E had the opposite effect (45% ± 27 increased, $P<0.001$) (Fig. 7B). Both CAT and NAC attenuated $CdCl_2$ induced HSP70 induction (reduced by 40% ± 6, $P<0.005$, 78% ± 6, $P<0.001$, respectively) (Fig. 7C). CAT and MnTBAP attenuated $CdCl_2$ induced HMOX-1 (reduced by 50% ± 5, $P<0.005$, 22% ± 17, $P<0.05$, respectively), while NAC abolished HMOX-1 induction (reduced by 97% ± 1, $P<0.001$) (Fig. 7D).

Discussion

The current study investigated the effect of Cd on airway epithelium, using Calu-3 cells as an *in vitro* model of human bronchial epithelial cells. We consider Calu-3 cells to be suitable for this purpose as they form tight junctions, produce mucous and express many of the characteristics of native epithelium, despite being derived from a human bronchial carcinoma [29]. Furthermore, culturing these cells at the air interface improves differentiation and better simulates the *in vivo* situation [18, 20, 21, 23].

In the present study we demonstrate that $CdCl_2$ exposure results in the disruption of barrier function at sub-cytotoxic concentrations. Additionally, barrier function disruption was more pronounced when $CdCl_2$ was applied to the basolateral surface of the cells. This disturbance in barrier function was associated with a redistribution of E-cadherin, a disrupted expression of ZO-1 and a loss of cell polarity. It has been suggested that Cd

may interfere with the extracellular Ca^{2+} binding sites on the E-cadherin protein located at the basolateral side of the cells [30]. Since E-cadherin plays a critical role in the development and maintenance of junctional complexes, the interference by Cd potentially disrupt cadherin dependent cell-cell junctions [31-35]. A recent *in vivo* study showed that Cd induced pulmonary oedema is associated with alterations in the localization of E-cadherin and VE-cadherin [36].

Beside the effect at the barrier level, it is known that Cd can activate transcription of a number of stress genes such as metallothionein, heat shock proteins, and heme oxygenase-1, which are involved in Cd detoxification, cellular protection, and lung defence against oxidant-induced injury [9, 10, 12-14, 37, 38]. In various cell models, it has been previously shown that low doses of Cd markedly increased the expression of MT1X, HMOX-1 and HSP70 [12, 39-47] suggesting their usefulness as biomarkers for evaluating the toxicity of Cd at low doses and the ability of the cells to respond to such stress [15]. Our results showed that apical treatment strongly induced MT1X at non cytotoxic concentrations, with only a slight induction of HMOX-1 and no effect on HSP70 expression. Whilst basolateral Cd exposure resulted in a stronger induction of HMOX-1 and MT1X and also induced HSP70 expression. These results suggest that Cd may be taken up more efficiently at the basolateral side of the cell. This may be due either to specific basolateral transport of Cd into the cells [48], or due to apical mucous sequestering of Cd and thereby decreasing the available concentration.

Cd can replace iron and copper in various proteins, leading to an increased amount of free ions which generates oxidative stress [8, 49]. Moreover, Cd interferes with thiol groups, and causes a depletion of endogenous intracellular antioxidants such as glutathione (GSH) resulting in a production of ROS (for reviews: [4, 5, 7, 8]). Here we tested the effects of four anti-oxidants on Cd induced mRNA expression and Cd induced barrier dysfunction. With the exception of Vitamin E, antioxidant treatment protected against Cd induced HMOX-1 and HSP70. This demonstrates that the increased expression of HMOX-1 and HSP70 is due to oxidative stress. However, only NAC caused a reduction in MT1X expression. The antioxidant activity of NAC is thought to be due to the thiol containing motif which acts as a precursor for GSH synthesis [50, 51]. However, NAC can also act as a direct Cd chelator [52] and thus may prevent certain modes of Cd interaction. Thus, since only NAC protected against Cd induced MT1X, it is likely that MT1X induction is in response to intracellular Cd concentration predominately. Also NAC totally abolished the Cd induced HSP70 and HMOX-1 response, thus demonstrating an anti-oxidant effect of NAC in addition to Cd chelation.

From the four antioxidants tested only NAC co-incubation provided any protection from Cd induced barrier damage. Moreover, NAC completely protected the cells from this effect. This result gives weight to the hypothesis that Cd acts to interfere with junctional processes such as E-Cadherin and can cause barrier function disruption due to extracellular binding. These results are in accordance with previous studies showing *in vitro* the protective effect of NAC against Cd induced cytotoxicity, ROS production, and up-regulation of stress related genes [44, 46, 47, 53-57]. A recent *in vivo* study showed that administration of NAC to rats instilled with $CdCl_2$, prevented collagen deposition and limited pulmonary fibrosis [58]. Whether the dominant protective effect of NAC is due to binding of Cd with its sulfhydryl group, or due to its role as a GSH precursor, still remain to be demonstrated, but our results would support a dual protective effect.

In conclusion, $CdCl_2$ exposure resulted in a destruction of Calu-3 barrier function at non-cytotoxic concentrations and this effect was more potent when exposure was basolateral. $CdCl_2$ also induced the expression of MT1X, HMOX-1 and HSP70 and again basolateral administration was more sensitive. Co-administration of antioxidants could attenuate Cd induced HMOX-1 and HSP70 expression, while NAC completely prevented this induction and also prevented barrier collapse. NAC potentially chelates Cd thus supporting the hypothesis that barrier function disruption is due to a direct effect of Cd on adherens junction proteins. The developed Calu-3 model will be useful for the further elucidation of pathways leading to pulmonary toxicity due to chemical insult and thereby aiding the reduction, refinement and replacement (3Rs) of animal experiments for inhalation toxicity [59].

Acknowledgements

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